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SOME BIOLOGICAL AND PHYSICAL PROPERTIES OF LASER DEPOSITED HYDROXYAPATITE BASED FILMS

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Abstract

The preliminary results of biological and physical tests of hydroxyapatite thin films deposited on dental implants by a new technology with the KrF excimer laser ablation method were evaluated. Biological and physical properties were studied and analyzed by the lymphocyte proliferation test and scanning electron microscopy (SEM), X-ray analysis, Rutherford backscattering analysis (RBS) and particle induced X-ray emission (PIXE) methods. Ten bioceramic films from 45 samples had very good physical and biological properties. Creation of hydroxyapatite thin films with laser ablation can have a positive effect on adhesion of the film and protection for corrosion.

Key Words: Dentistry, implants, bioceramics, hydroxyapatite, laser.

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Introduction

One of the basic criteria in evaluating implants is their biotolerance, which provides conditions for acceptance by host tissues. Bioceramics show a high tolerance towards human body tissues, which is a precondition for success in clinical use. The group of bioceramics also includes hydroxyapatite and its crystalline phases (Ducheyne and De Groot, 1981; Klein *et al.*, 1983; Winter *et al.*, 1981).

The aim of the present study was to create a new coat for dental titanium alloy implants which can protect the intraosseal part of the implant against corrosion (Reclaru and Meyer, 1994). We wanted to find the optimal bioceramic film with good adhesion and biotolerance without resorption of the surface. Hydroxyapatite-based bioceramic material deposited as a thin film on titanium substrates by laser ablation was used for this purpose. This thin film should have strong bonds to the titanium substrate and sufficient biotolerance. Therefore, we checked the physical conditions and properties (Jelinek *et al.*, 1995) of these films and evaluated their biological acceptance.

In general, for an evaluation of the biotolerance of materials, the following tests can be found in the literature: the systemic cytotoxicity test, the macrocontact toxicity test, the hemolytic test, the blastic transformation test, the combined test of cytotoxicity, and other tests (ISO, 1984; Procházková and John, 1986). The international standard methods were given in International Standard ISO 10993-5, Biological Evaluation of Medical Devices (ISO, 1992).

Materials and Methods

Materials

Bioceramic samples were supplied by the Institute of Physics, Czech Academy of Sciences, Prague, Czech Republic. The specimens consisted of thin films of hydroxyapatite on a titanium substrate. The hydroxyapatite phase was deposited from a hydroxyapatite target on titanium by laser ablation. By changing the conditions of

deposition a wide range of film properties could be created (Jelinek *et al.*, 1994).

For cell cultures the RPMI 1640 medium (ÚMG ČAV, Prague, Czech Republic) was used (Procházková and John, 1986).

Mononuclear cells were isolated from the heparinized blood of healthy donors.

Cells were stimulated by phytohemagglutinin (PHA; Difco, Detroit, MI, USA) at a concentration of 1 mg/ml in the RPMI 1640 medium.

The control solution (RPMI supplemented medium) contained RPMI 1640 medium with all additives (as listed in the text) but without any sample (and therefore, this solution cannot affect lymphocyte function).

Methods

Sample preparation. Thin films of bioceramic were deposited on flat titanium substrates by laser ablation. The experimental set-up consisted of a KrF excimer laser (Lambda Physik LPX 200) and a stainless steel interaction chamber where the hydroxyapatite target and substrates were placed. A laser beam (wavelength 248 nm, pulse duration 30 ns, repetition rate 10 Hz) was focused on the target. Evaporated material from the target was stoichiometrically transferred to a substrate. The films were deposited in various atmospheres (vacuum, water vapor, or a mixture of Ar/water vapor), at various temperatures of the substrate (200-760°C) and at different energy densities of the laser beam on the target (3 $J.cm^{-2}$ and 13 $J.cm^{-2}$) (Jelinek *et al.*, 1994, 1995). The Ca/P ratio was determined by Rutherford backscattering (RBS) and proton-induced X-ray emission (PIXE) techniques. The standard RBS measurements were performed using 1.3 MeV ^4He ions. The PIXE analyses were performed with 2.5 MeV protons. A Bragg-Bretano goniometer (Cu Kα radiation, 200 mA, 50 kV) was used for X-ray diffraction (XRD) measurements. The film topography was observed by scanning electron microscopy (SEM) using a 10 kV electron beam.

Immersion of bioceramic samples into the medium. The specimens were rinsed in distilled water and wrapped in aluminum foil. Subsequently, they were sterilized in a hot air sterilizer at 180°C for 30 minutes. Samples were placed in 1 ml multiwell dishes and incubated for seven days in 0.5 ml of RPMI 1640 medium at pH 7.3.

Cells. Mononuclear cells were isolated from 50 ml heparinized (5 U/ml) venous blood from one healthy donor. The blood was diluted with an equal volume of culture medium and covered with a Ficoll-Paque gradient with a density of 1.077 g/ml (Boyum, 1968). After that, blood was centrifuged at 400 *g* for 30 minutes and the mononuclear cell interface was carefully collected and washed three times in RPMI 1640 medium to which $NaHCO_3$ (ÚSOL, Prague, Czech Republic) was added to obtain pH 7.3 for cell culture. The lymphocytes were suspended at a concentration of 2 x 10^6 cells/ml in RPMI 1640 medium with HEPES buffer (4 x 10^{-3}M, Sigma Chemical Co., St. Louis, MO, USA), 5% calf serum (BIOCOM, Brno, Czech Republic) and 0.1 ml gentamicin (40 mg/ml) (Lek, Ljubljana, Slovenia).

Tests were carried out according to the following schedule: 3 multiwell dishes (Microwell Plate, Nunc, Roskilde, Denmark) were used, i.e., a total of 288 wells, and 100 μl cell suspension was added to each well. To 12 wells, 50 μl of supplemented RPMI 1640 medium were added as a control, to each of the remaining wells (276 wells), 50 μl eluate was added (experimental). To half of both experimental (138 wells) and control wells (6 wells), 50 μl PHA were added (stimulated cells). To the other half of experimental and control cells, 50 μl RPMI 1640 supplemented medium was added (non-stimulated cells).

The cells were cultured for 48 hours at 37°C in a humidified atmosphere with 5% CO_2 (Procházková and John, 1986).

Evaluation

Lymphocyte activity was evaluated by a proliferation test based on the activity of stimulated and non-stimulated cells. This ratio was called the stimulation index SI. The stimulation index of pure titanium was 1.45.

Radiolabelled ^3H-thymidine incorporation was used to check proliferation. Fifty μl medium containing 3.7 MBq ^3H-thymidine was added to each well. All cells were cultured for 4 hours at 37°C in a humidified atmosphere with 5% CO_2. Subsequently, specimens were harvested on a glass paper (Whatman GF/A, Maidstone, England), dried and transferred into small glasses with 3 ml scintillation solution prepared from 0.2 g POPOP (1,4-bis/2,5-phenyloxazolyl/benzene) and 5 g PPO (2,5-diphenyloxazol) dissolved in 1 l of toluene (all from Lachema, Brno, Czech Republic). The activity of the specimens was measured by a beta counter for 1 minute.

The following characteristics of deposited films were determined: stoichiometry (by RBS and PIXE), crystalline phases (by XRD), surface morphology (by SEM), and Knoop microhardness of films and films-substrate adhesion (Jelínek *et al.*, 1994, 1995).

Results

Proliferation test

The values determined for the 46 samples are presented in Table 1. The basic value for their classification was the stimulation index of pure titanium, which was 1.45. Samples with a lower index were considered to

Table 1. Results of the proliferation test

sample	f_{stim}	$f_{non\text{-}stim.}$	SI
6	2938	2562	**1.15**
7	847	1103	**0.77**
8	2156	2365	**0.91**
9	847	476	***1.78**
10	2252	2665	**0.85**
11	2032	625	***3.12**
12	1612	1951	**0.83**
13	873	2524	**0.35**
14	1986	2371	**0.84**
15	904	1062	**0.85**
16	3085	3998	**0.77**
17	472	2390	**0.20**
18	2018	1158	***1.74**
19	442	596	**0.74**
20	323	1547	**0.21**
22	423	283	***1.49**
23	1298	1722	**0.75**
24	177	300	**0.59**
25	1706	2980	**0.57**
26	341	638	**0.53**
27	2255	2169	**1.04**
28	315	999	**0.32**
29	3222	2650	**1.22**
30	340	906	**0.38**
31	3242	2243	**1.45**
32	458	504	**0.91**
33	2175	1836	**1.18**
34	637	1264	**0.51**
35	3707	1268	***2.92**
36	1329	1130	**1.18**
37	718	714	**1.01**
38	2377	3387	**0.70**
39	352	796	**0.44**
44	1916	3869	**0.49**
45	646	382	***1.69**
46	1096	4134	**0.27**
47	401	239	***1.68**
48	634	1954	**0.32**
49	1736	806	***2.10**
50	2092	3842	**0.55**
51	433	477	**0.91**
52	1432	3320	**0.43**
53	848	422	***2.01**
54	2047	733	***2.79**
Ti	1884	1271	**1.45**

SI = Stimulation Index (ratio of stimulated to non-stimulated cells).
*: values exceeding the SI of Ti.

have worse tolerance and probably inferior acceptability of host tissues. Specimens with a higher index are listed in Table 2 with their physical properties. Sample 11 had the highest stimulation index (3.12).

Physical tests

X-ray spectra of hydroxyapatite films, similar to those of hydroxyapatite targets, were observed mainly for deposits in the mixture of Ar/water vapor and at high substrate temperatures (600°C-760°C). Preferential hydroxyapatite film orientations were found to be changed depending on the conditions of deposition from (300) to (002) and (112). In addition to HA peaks, peaks of $Ca_4O(PO_4)_2$ or phosphate phases were observed. The Ca/P ratio in the films was dependent on the substrate deposit temperature. Values close to the optimum (1.67) were reached with lower deposit temperatures and higher density laser beam energy. The morphology of the deposited films was generally good for all deposit conditions, but smoother surfaces were obtained at lower deposit temperatures (Jelinek *et al.*, 1994, 1995).

Surface morphology (SEM)

As was mentioned above, sample 11 had the best stimulation index, but after the lymphocyte proliferation, test blisters and resorbed areas of the surface were observed. Cracked bioceramic films were found in sample 45, and in samples 9 and 47 spots of film were detected. These bioceramic films had very good physical and biological properties but showed more extensive surface changes. Samples 53 and 54 had good physical properties, a better stimulation index than pure titanium and optimal surface morphology. Typical examples of surface morphology are presented in Figures 1 to 6.

Discussion

Hydroxyapatite is commonly used as a coating material on some types of clinical implants as a protection against corrosion and as a permanent isolation between the metal part and the bone. The protection is based on the close relation between the hydroxyapatite-coat and bone (Knöfler, 1994; Oguchi, 1994).

Physical properties of bioceramic materials are of principal importance for the optimal connection (adhesion) of hydroxyapatite and the titanium implant (Schliephake *et al.*, 1995). The conditions for creating thin hydroxyapatite films can be changed using a new technology using the KrF excimer laser ablation method (Jelinek *et al.*, 1994, 1995). From our results, it can be concluded that the alteration of surface structures after contact with RPMI 1640 medium (at pH 7.3) has a direct influence on the biological properties of samples, because the free titanium surface can corrode. The

Table 2. Results of biocompatibility and comparable physical properties of layers and deposition conditions (stimulation index, film properties, deposition conditions).

Sample	SI	Ca/P	Crystalline Phases	T[°C]	Gas Environment	Pressure
9	1.78	3.33	HA, 4CP, 3CP, TiO_2	760	H_2O	2×10^{-3} mbar
11	3.12	2.27	HA, TiO_2	600	H_2O	2×10^{-1} mbar
18	1.74	2.17	-	500	H_2O + Ar	300 mTorr
22	1.50	2.44	-	500	H_2O	2×10^{-3} mbar
35	2.92	2.94	4CP, HA, CaO	760	H_2O	2×10^{-3} mbar
45	1.69	2.94	HA, CaO	600	H_2O	2×10^{-1} mbar
47	1.68	2.94	HA	760	H_2O	2×10^{-1} mbar
49	2.15	3.45	HA (am. + cr.), TiO_2	600	H_2O	8×10^{-2} mbar
53	2.01	1.82	HA, 4CP, TiO_2	600	H_2O + Ar	300 mTorr
54	2.79	2.33	HA, 4CP, TiO_2	760	H_2O + Ar	300 mTorr

HA = hydroxyapatite, 3CP = tricalcium phosphate, 4CP = tetracalcium phosphate, SI = stimulation index, am. = amorphous, cr. = crystalline.

reactivity of the implant surface can be measured by a proliferation test based on the activity of stimulated and non-stimulated cells. This ratio was called the stimulation index. For pure titanium, this index was 1.45. It can be assumed that samples with a lower index have worse tolerance and probably worse acceptability of host tissues. A higher index does not, however, guarantee a better quality of the hydroxyapatite film. The biological properties partly depend on the quality of the surface after the biological tests.

For evaluation of material biotolerance, tests such as the systemic cytotoxicity test, the macrocontact toxicity test, the hemolytic test, the blastic transformation test, and the combined test of cytotoxicity were developed (Procházková and John, 1986; ISO, 1984). However, some of these tests do not answer the question whether alterations in cell function have taken place, although these changes may play an important role in implant/host tissue reactions. T-lymphocytes identify foreign bodies such as metals, plastics, and biological materials, and initiate the reaction of the immune system against these substances (Procházková and John, 1986). For this reason, the present preliminary study was set up to change the function of T-lymphocytes, because their proliferative activity increases after contact with antigens or foreign materials (Stejskal *et al.*, 1986). We used mononuclear cells isolated from the peripheral blood of a healthy donor and stimulated by PHA, and compared their reactions with that of non-stimulated cells (Procházková and John, 1986). Eluate from bioceramic samples into the medium was added to part of the lymphocyte samples. We assumed that samples without any influence on the lymphocytes would show the same reaction as cells in wells free of eluate, with only PHA present, but that samples influenced by the medium would change T-lymphocyte function (Stejskal *et al.*, 1986).

Bioceramic surface quality is one of the most influential factors for evaluation (Osborn, 1985; Oguchi, 1994). After the lymphocyte proliferation test, the surface can be irreversibly destroyed. Blisters and resorbed areas of surface, cracked bioceramic films, or spots of film were observed after the test. As mentioned above, these bioceramic films had very good physical and biological properties but they were associated with more extensive changes of the surface. Hard tissue response to degradation of hydroxyapatite ceramic coated implants (68%) was described by Søballe (1993). Creation of hydroxyapatite thin films with laser ablation can have a positive effect on adhesion of the film and on protection for corrosion.

Figures 1-6 (facing page). Scanning electron micrographs.

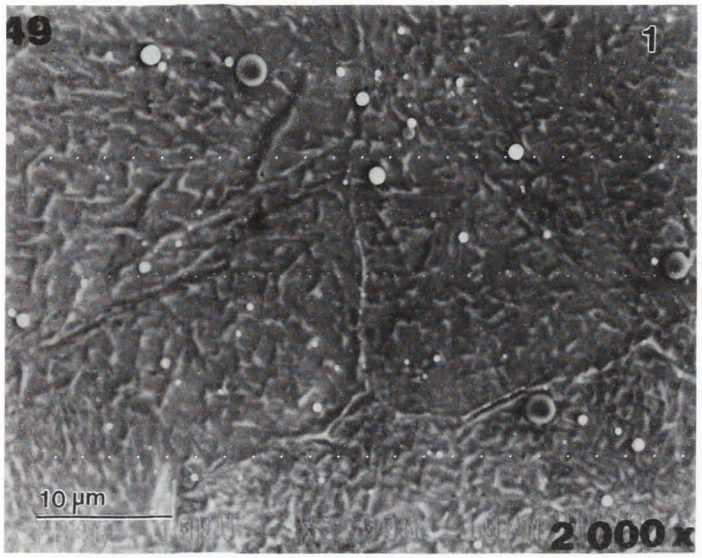
Figures 1 and 2. Sample 49: before the proliferation test (**Fig. 1**), and showing cracks after the proliferation test (**Fig. 2**).

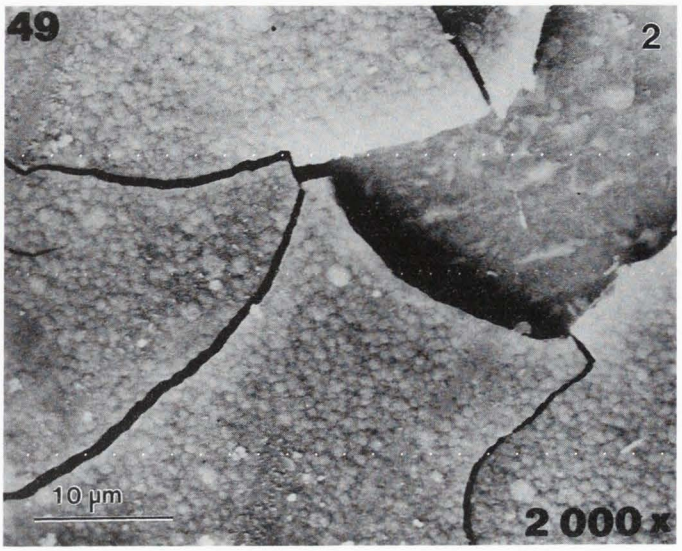
Figure 3. Sample 47 before the proliferation test.

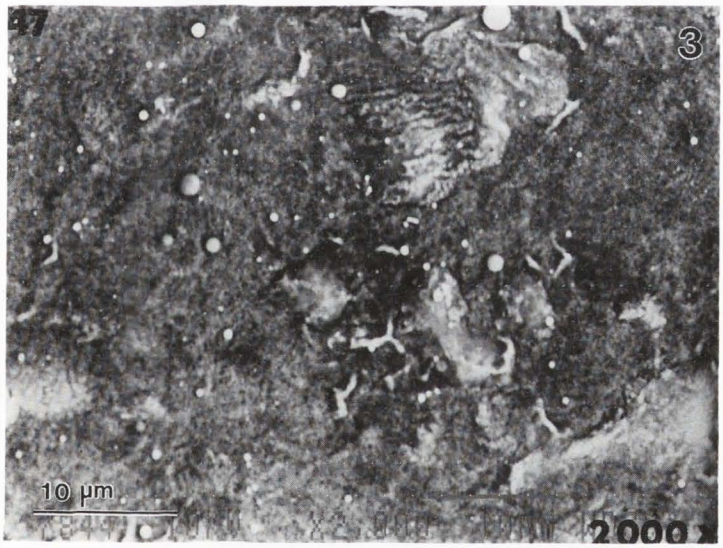
Figure 4. Point resorption in the shape of small bubbles (sample 47).

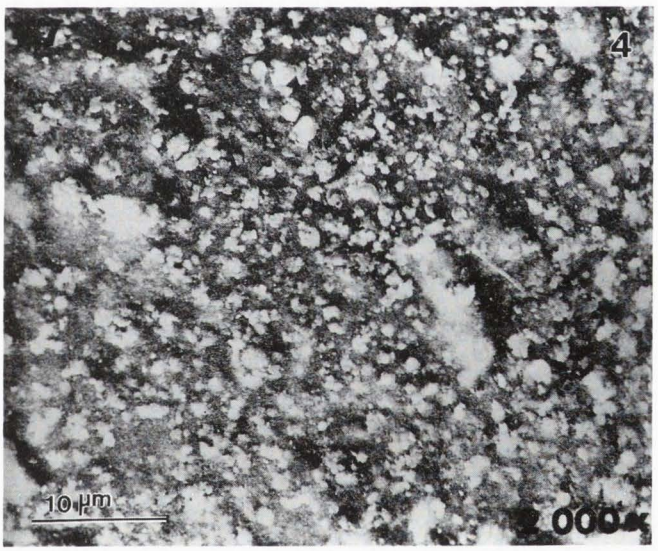
Figure 5. Sample 53 before the proliferation test.

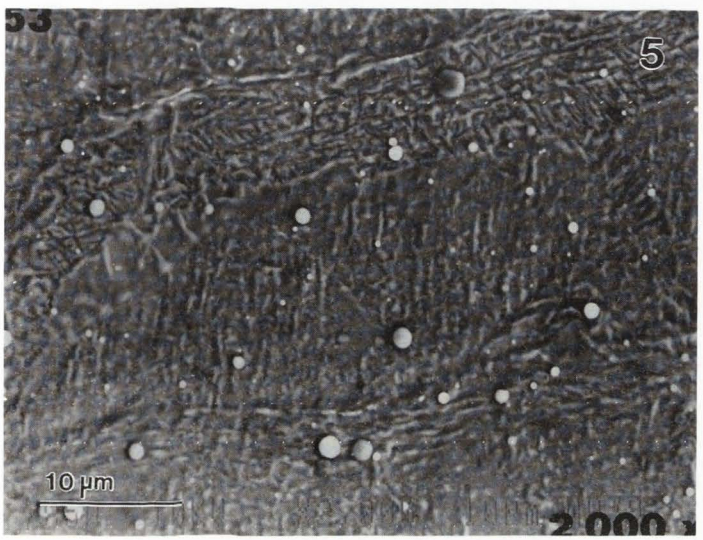
Figure 6. Optimal surface conditions from the point of view of surface stability, sample 53.

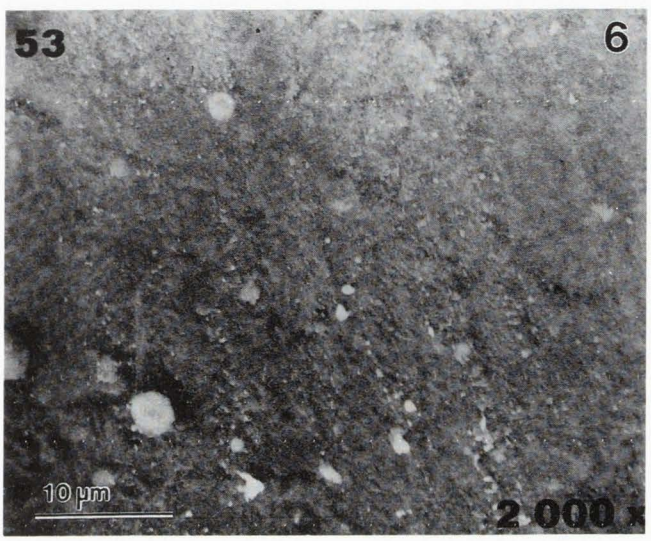

49
1
10 µm
2 000 x


49
2
10 µm
2 000 x


47
3
10 µm
2000 x


4
10 µm
2 000 x


53
5
10 µm
2 000 x


53
6
10 µm
2 000 x

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no **Discussion with Reviewers**.